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VASOPRESSIN AND V1BR GENE EXPRESSION IS INCREASED IN HYPOTHALAMIC PVN OF BORDERLINE HYPERTENSIVE RATS

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Complete List of Authors:	Savić, Bojana; University of Belgrade Faculty of Medicine, Institute of Pharmacology, Clinical Pharmacology and Toxicology Martin, Andrew ; University of Bristol Medical School, Translational Health Sciences, Dorothy Hodgkin Building, Souza Mecawi, Andre; Federal University of Sao Paulo Paulista Medical School, Laboratory of Neuroendocrinology, Department of Biophysics Bukumirić, Zoran; University of Belgrade Faculty of Medicine, Institute for Medical Statics and Informatics Antunes-Rodrigues, José ; University of Sao Paulo Faculty of Medicine of Ribeirao Preto, Department of Physiology Murphy, David; University of Bristol Medical School, Translational Health Sciences, Dorothy Hodgkin Building Šarenac, Olivera; University of Belgrade Faculty of Medicine, Institute of Pharmacology, Clinical Pharmacology and Toxicology Japundžić-Žigon, Nina; University of Belgrade Faculty of Medicine, Institute of Pharmacology, Clinical Pharmacology and Toxicology
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VASOPRESSIN AND V1BR GENE EXPRESSION IS INCREASED IN HYPOTHALAMIC PVN OF BORDERLINE HYPERTENSIVE RATS

Bojana Savić[1], Andrew Martin[2], Andre Souza Mecawi[3], Zoran Bukumirić[4], José Antunes-Rodrigues[5], David Murphy[2], Olivera Šarenac[1], Nina Japundžić –Žigon[1]

[1]Institute of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade, Belgrade, RS.

[2]Bristol Medical School: Translational Health Sciences, Dorothy Hodgkin Building, University of Bristol, Bristol, BS1 3NY, UK.

[3]Laboratory of Neuroendocrinology, Department of Biophysics, Paulista Medical School, Federal University of São Paulo, São Paulo, Brazil

[4]Institute for Medical Statics and Informatics, Faculty of Medicine, University of Belgrade, Belgrade, RS.

[5]Department of Physiology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.

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Corresponding author: Prof Dr Nina Japundžić-Žigon

E-mail 1: nina.zigon@med.bg.ac.rs; E-mail 2: nina.japundzic@gmail.com

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ABSTRACT

Vasopressin (VP) is a neurohypophyseal peptide best known for its role in maintaining osmotic and cardiovascular homeostasis. The main sources of VP are the supraoptic and paraventricular (PVN) nuclei of the hypothalamus, which co-express the vasopressin V1a and V1b receptors (V1aR and V1bR). Here we investigate the level of expression of VP and VP receptors in the PVN of borderline hypertensive rats (BHRs), a key integrative nucleus in neuroendocrine cardiovascular control. Experiments were performed in male BHRs and Wistar rats (WR) equipped with radiotelemetry device for continuous hemodynamic recordings, under baseline conditions and after saline load without or with stress. The autonomic control of the circulation was evaluated by spectral analysis of blood pressure (BP) and heart rate (HR) variability and baro-receptor reflex sensitivity (BRS) using the sequence method. Plasma VP was determined by radioimmunoassay, and VP, V1aR and V1bR gene expression by RT-qPCR. Under baseline conditions, BHRs had higher BP, lower HR and enhanced BRS in respect to WRs. BP and HR variability was unchanged. In the PVN, overexpression of VP and V1bRs genes were found and plasma VP was increased. Saline load downregulated V1bR mRNA without affecting VP mRNA expression, plasma VP and the BP. Adding stress increased BP, BP and HR low frequency sympathetic spectral markers, decreased plasma VP without altering the level of expression of VP and VP receptors in PVN. It follows that overexpression of VP and V1bR in the PVN is a characteristic trait of BHR, and that sympathetic hyperactivity underlies stress-induced hypertension.

KEY WORDS: Vasopressin, vasopressin receptors, borderline hypertension, stress, hypothalamic paraventricular nucleus

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INTRODUCTION

Vasopressin (VP) is a neurohypophyseal peptide hormone engaged in an abundance of physiological and behavioural regulatory processes, but it is best known for its importance in maintaining osmotic and cardiovascular homeostasis. The main sources of VP in the brain are the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus [1,2]. It is well established that PVN is a key integrative site for neuroendocrine and autonomic responses relevant for cardiovascular regulation. Large magnocellular neurons (MCNs) of the PVN synthesize VP, which is axonally transported to the neurohypophysis for systemic secretion [1,2]. In the periphery, VP fosters water reabsorption in the kidneys and vasoconstricts blood vessels to increase peripheral resistance supporting circulation [3]. Smaller parvocellular neurons of the PVN comprise a neuroendocrine portion which synthesizes VP and releases it in to the hypophyseal portal circulation, and a pre-autonomic neuronal subgroup responsible for the sympathetic nervous system activation that contributes to hypertension [4]. These latter neurons modify sympathetic outflow by virtue of projections to the brain stem autonomic nuclei in the rostroventral part of the medulla (RVLM vasomotor neurons) and the intermediolateral column of the spinal cord (IML), enabling them to modulate cardiovascular and renal homeostasis [5]. VP is also released from somata and dendrites of MCNs where it performs auto-regulation via specific co-localised vasopressin receptors (V1aR and V1bR) [6,7]. Osmotic challenges have been shown to modify the expression of V1aRs, modulate VP release and sympathetic outflow in normotensive rat strains [8-10]. V1bRs in the PVN were also found to be implicated in the blood pressure (BP) increase caused by VP microinfusion into the PVN [11]. However, the possible roles of VP, V1aRs and V1bRs in the PVN in the pathogenesis of borderline hypertension has not been elucidated.

For Review Only

We hypothesized that changes in the gene expression of V1aR and V1bR receptors in PVN may modulate VP gene expression and release and thus contribute to the genesis of borderline hypertension in rats (BHR) and their susceptibility to develop overt hypertension when exposed to repeated environmental stressors [12]. Therefore, we evaluated the gene expression of VP, V1aR and V1bR in the PVN of BHRs as well as plasma VP and cardiovascular autonomic control under baseline physiological condition and when submitted to a 24-weeks long salt load with and without repeated stress.

For Review Only

METHODS

All experimental procedures in this study conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, as well as UK Animal Act 1986. The experimental protocol was approved by the School of Medicine University of Belgrade Ethics review board (# 323-07-04083/2016-05/7), and all studies involving animals comply with the ARRIVE guidelines for reporting experiments involving animals [13,14].

Animals

Two strains of rats, Wistar rats (WR) and BHRs, were included in the study. BHR were produced in a local animal facility as an F1 generated by crossbreeding spontaneously hypertensive rat (SHR) females and normotensive WR males. All animals were matched regarding their age (12 weeks old) and weight (280-330 g), and were housed in a controlled environment (12 h/12 h light-dark cycle, temperature 21±2◦C and humidity 65±9%) with access to standard food pellets (0.2% sodium content; Veterinarski zavod, Subotica, Republic of Serbia) and tap water or 0.9% saline solution *ad libitum*. The number of rats enrolled in experimentation was determined in 'Power Sample Size Calculation' software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize) for power of 90% and type I error probability of 0.05.

Implantation of radiotelemetry probe in abdominal aorta

Animals were anesthetized using a combination of ketamine (100 mg•kg^{-1}, I.M.) and xylazine (10 mg•kg^{-1}, I.M.). The depth of anesthesia was verified by the lack of corneal and pedal reflexes, at the beginning and throughout the surgery. Rat body temperature was

For Review Only

maintained by a heating pad (Harvard Apparatus, Holliston, MA, USA). Following medial abdominal 3 cm-long incision and intestine retraction, a catheter tip of radiotelemetry device (TA11-PA C40; DSI, Transoma Medical, St Paul, MN, USA) was inserted into aorta, fixed with 3M Vetbond™ and tissue cellulose patch (DSI, Transoma Medical). Perioperatively, rats were treated with gentamicin (25 mg•kg^{-1}, i.m.) and carprofen (5 mg•kg^{-1} daily, s.c.) to prevent secondary bacterial infections and to reduce pain, respectively. Postoperatively, animals were housed individually (30 × 30 × 30 cm Plexiglas cage) in a separate surrounding under controlled laboratory conditions. They were monitored every day until full recovery.

Experiment design

Fourteen days following surgery rats were fully recovered and randomly allocated in four groups subjected to different protocols. In group 1, Wistar rats drinking tap water were kept under baseline physiological conditions for 24 weeks. In group 2, BHRs drinking tap water were kept under baseline conditions for 24 weeks. In group 3, BHRs were drinking 0.9% saline solution for 24 weeks, while in group 4, BHRs were drinking 0.9% saline solution and were exposed to repeated heterotypic stressors to minimize conditioning, also for 24 weeks. Heterotypic stress was produced by applying alternating stress batteries. Battery 1 lasted 4 weeks: crowding stress (rats were housed in a reduced living space: 70 cm2/100g body weight) plus shaking stress (200 cycles/min for 30 min), for 6 days per week. Battery 2 lasted 6 weeks: isolation in opaque Plexiglas cages (300 cm2/100g body weight) plus inclination at 40º for 60 minutes followed by 2-min-long air jet directed to the top of rats′ head, for 6 days per week. Battery 1 was repeated for 3 times, and battery 2 was repeated for 2 times, in total for 24 weeks. On each seventh day (day without exposure to stress), SBP, DBP, HR, BRS and spectral parameters were monitored and shown after completion of each stress battery.

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Cardiovascular signal processing and analysis

Arterial BP was digitalized at 1000 Hz in Dataquest A.R.T. 4.0 software, (DSI, Transoma Medical). Systolic (SBP) and diastolic BP (DBP) and pulse interval (PI) or its inverse heart rate (HR) were derived from the arterial pulse pressure wave as maximum, minimum and dP/dt_{max} inter-distance, respectively.

Evaluation of the spontaneous baroreceptor reflex by the sequences method

The method is explained in detail elsewhere [15]. A stream of, at least four, consecutively increasing or decreasing SBP values and PI values delayed by three, four or five beats with respect to SBP, were considered as baro-receptor reflex sequence [16]. The following parameters were calculated as follows:

- *Baroreceptor reflex sensitivity* (BRS, ms•mmHg^{-1}) as linear regression coefficient averaged over all identified sequences (PI = BRS•SBP + const., where fitting of the curve is done in a least square method);
- *Baroreceptor effectiveness index* (BEI) as ratio of the number of SBP-PI sequences versus number of SBP ramps
- *Operating range* (OR) as a rectangle surface covering 95% of SBP-PI sequences.
- *Set Point* (SP) as a median of all SBP-PI sequences.

Spectral analysis of BP and HR

Spectral analysis of SBP, DBP and HR based on fast Fourier transformation, was performed after linear de-trending, nine-point Hanning window filtering and resampling at 20Hz [17]. Spectra were obtained on 30 overlapping 2048 points time series of SBP, DBP and HR. The BP (mmHg^2) and HR (bpm^2) spectral powers were calculated for the whole spectrum (0.0195–3Hz) and in the three frequency ranges: very low frequency (VLF, 0.0195–0.195 Hz),

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low frequency (LF, 0.195–0.8Hz) and high frequency (HF, 0.8–3Hz). The LF SBP and LF DBP and LF/HF HR are recognized autonomic markers in both clinical [18,19] and experimental practices [20]

Blood and tissue collection for evaluation of plasma VP and mRNA expression of VP, V1aR and V1bR in the rat PVN

At the end of the last recording session rats were euthanized using guillotine (Harvard Apparatus, Holliston, Massachusetts, USA) for fast decapitation. The blood was collected from the trunk in chilled plastic tubes with heparin and the brains were removed and snap frozen with powdered dry ice. Plasma was obtained after centrifugation (20 min, 1600 *g*, 4°C) and stored at -80 °C for up to six months before the hormone extraction and lyophilisation. Lyophilised plasma was stored at -20 °C for six months until the radioimmunoassay.

The specific antibody for VP radioimmunoassay was obtained from Peninsula (T4561, San Carlos, CA, USA). The VP extraction and measurements were performed by using the specific radioimmunoassay techniques that were previously described by Husain *et al* [21]. 1973. The VP measurement was performed in duplicate. The radioimmunoassay sensitivity range was from 0.4 to 32.0 pg/ml and intra- and inter-assay coefficients of variation were 7.9–9.75%, respectively.

By using a cryostat (Leica Microsystems CM1900, Leica Microsystems, Nussloch GmbH, Nussloch, Germany), 60 µm caudal-rostral brain slices were taken and stained with Toluidine blue (1% in 70% ethanol, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) for mapping and verification of the hypothalamus [22]. Both left and right PVN unstained tissue were punched taking 1 mm in diameter, using a 15-G Sample Corer (Fine Science Tools Inc., Foster City, CA, USA, cat.no 18035-01), and stored in RNase-free tubes at −80°C.

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RNA extraction

Tissue samples were homogenized in 1 mL TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) by vortexing for 30 s. Samples were centrifuged at 12000 rpm for 12 minutes at 4°C. Supernatants were aspirated by pipette into new tubes, while the pellet of cellular debris was discarded. Extraction was performed with 200 µL chloroform (Sigma-Aldrich Co. Ltd.), added to each sample followed by vortexing. After 2-3 minutes of standing at room temperature, samples were centrifuged for 15 min, at 12 000 rpm, at 4°C. Aqueous upper phase, containing the RNA were decanted into fresh tubes; without disturbing the interphase or lower organic phase in which both the DNA and proteins were located. Total RNA from aqueous phase was then precipitated with 1 volume (~350 µL) of ethanol (EtOH 100% v/v), and purified using the RNeasy Mini Kit (Qiagen, Qiagen Ltd., Manchester, UK) according to manufacturer's protocol.

cDNA synthesis

cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen, Qiagen Ltd., Manchester, UK) using 100 ng of input RNA. cDNA samples were then diluted to a concentration of 2 ng·µL−1 for use in real time qPCR.

Real time quantitative polymerase chain reaction (qPCR) and data analysis

Primers for the housekeeping gene RPL19 (Ribosomal protein L19: 5′-GCGTCTGCAGCCATGAGTA-3′ and 5′-TGGCATTGGCGATTTCGTTG-3′) were used to allow normalization of receptor expression between samples. Other primers for rat genes used in this study were VP (5′-TGCCTGCTACTTCCAGAACTGC-3′ and 5′-AGGGGAGACACTGTCTCAGCTC-3′) synthetized by Eurofins MWG Synthesis GmbH, Ebersberg, Germany, V1aR (Rn Avpr1a 1 SG QuantiTect Primer Assay, QT00402990,NM

For Review Only

053019) obtained from Qiagen (Qiagen, Qiagen Ltd., Manchester, UK), V1bR (5′-TGCCACATTCCTGGAGTACCT-3′, 5′-AGGACGGTTAACCAAGTAG TGAGATG-3′) provided by Invitrogen. All primers were designed using GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Analysis was performed using an Applied Biosystems Viia7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and processed using ViiA 7 Software v1.1 (Applied Biosystems, Foster City, CA, USA). The Δ cycle threshold (ΔCt = sample gene Ct − housekeeper gene Ct) was calculated for each sample and then exponentiation was applied to each sample (Power 2).

Drugs

Ketamine (Ketamidor), xylazine (Xylased®) and carprofen (Rimadyl®) were purchased from Marlofarma (RS). Gentamicin injections were obtained from Hemofarm (Vršac, RS).

Statistics

Results are presented as mean value ± standard error of the mean. BP, HR, spectral and baroreceptor reflex parameters statistical differences between WR and BHR strain were estimated using Student's T-test (two-sample T-test) in SPSS Statistics v.20 software (IBM Corporation, NY, USA). qPCR data was analysed by One way ANOVA followed by Tukey post hoc, while data collected during the follow-up period were assessed by ANOVA for repeated measures within experimental groups in SPSS Statistics v.20 software. Mixed Effect Regression model was used to compare data between different experimental groups overtime in R-3.5.0 software (The R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was considered at $p < 0.05$.

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RESULTS

Plasma VP and expression of the VP, V1aR and V1bR genes in the PVN of WR and BHR under baseline conditions and following saline loading with or without stress

Plasma VP was increased in BHRs under baseline physiological conditions in respect to WRs. (Figure 1). BHRs exhibited a three-to-four-fold increase of VP and V1bR mRNA expression levels in the PVN as compared to WRs while V1aR mRNA levels did not change (Figure 1).

Exposure of BHRs to saline load did not affect plasma VP concentrations nor VP and V1aR mRNA levels, but reduced V1bR mRNA levels with respect to BHR controls kept under baseline conditions (Figure 1). Exposure of salt loaded BHRs to heterotypic stressors decreased plasma VP concentration compared to BHR controls and salt loaded BHRs. In stressed rats VP, V1aR and V1bR mRNA levels did not change and were comparable to BHR controls (Figure 1).

BP, HR, their short-term variability and the baroreceptor reflex in BHRs and WRs under baseline conditions

Under baseline conditions, BHRs exhibited higher SBP, DBP and lower HR values compared to WRs (Table 1). Spectral analysis of BP and HR short-term variability revealed no differences between the experimental groups (Figure 2). BRS and OR of BHRs were increased with respect to WRs under baseline physiological conditions and the baro-receptor reflex was re-set toward higher SBP values (Table 1).

For Review Only

BP, HR, their short-term variability and BRS in saline loaded BHRs with or without exposure to stress

Saline load alone did not affect SBP and DBP, but it decreased HR in respect to baseline values. In these rats BRS did not change overtime in respect to baseline values and in respect to BHR controls (Figure 3). Saline loaded BHRs exposed to stress showed a statistically significant increase of SBP over time with respect to baseline values and BHRs controls. DBP increase did not reach statistical significance. In these rats, BRS also increased whilst HR decreased over time in respect to baseline conditions (Figure 3).

In saline loaded BHRs autonomic spectral markers indicate a reduction of sympathetic influences on the blood vessels and the heart: LF-SBP and LF-DBP decreased over time with respect to baseline conditions, and LF-HR and LF/HF-HR were lower compared to BHR controls. In contrast, autonomic spectral markers LF-SBP and LF-DBP and LF/HF-HR were enhanced in saline loaded and stressed BHRs with respect to BHR controls (Figure 4).

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DISSCUSION

For the first time to our knowledge, we have described strain specific differences in VP and its receptors mRNA within the PVN of BHRs compared with normotensive controls. Under baseline conditions, BHRs exhibit elevated mean values of SBP, DBP, reduced HR and increased BRS, operating range and baroreceptor reflex resetting with respect to control WR. In BHRs qPCR analysis of the PVN revealed increased V1bR and VP gene expression and VP plasma concentration was increased. Saline load did not affect BP, BRS, plasma VP and VP gene expression in BHRs. However, V1bR gene expression decreased. When BHRs were exposed to saline load plus stress they developed overt hypertension and had decreased plasma VP. In these rats spectral markers reflecting sympathetic hyperactivity to blood vessels (LF-BP) and the heart (LF/HF HR) were enhanced. Neither VP nor VP receptor gene expression in the PVN were changed in these rats.

The BHR is the first filial offspring of crosses between SHRs and normotensive Wistar rats and thus possesses genetic information from both the normotensive and the hypertensive parent. The aetiology of borderline hypertension is not well understood and is different from genetic hypertension (SHR). Our present results suggest the involvement of VP in the pathogenesis of borderline hypertension. It has been documented previously that VP both centrally and peripherally contributes, but does not play a key role in the pathogenesis of genetic hypertension [23-28]. It has also been suggested that increased expression of VP mRNA in the PVN and SON is a constitutive trait of SHR, found in the pre-hypertensive stage in the SHR[28]. VP released from the magnocellular division of the PVN into the systemic circulation targets V2Rs in the collecting ducts of the kidneys to conserve water [29], and V1aRs in blood vessels to increase peripheral resistance. These effects of VP are important for support of the circulation and are lifesaving under hypovolemic and hypotensive conditions [30-32]. At a molar

For Review Only

level, VP has been shown to be the most potent vasoconstrictor [33]. However, when applied systemically to normovolemic and normotenisve rats, VP only moderately increases BP because it increases the BRS and more effectively decreases HR, which then more efficiently opposes increased peripheral resistance [34]. The effect of VP on the BRS was found to be mediated via the area postrema, a structure lacking a blood brain barrier, which is abundantly connected to the nucleus of the solitary tract (NTS), where baro-receptor reflex afferents terminate [35]. There is pharmacological evidence that both types of VP receptors, V1a and V2, are involved in BRS modulation in the rat area postrema [36-38]. Thus, in present experiments, increased expression of the VP mRNA in the PVN of BHR rats may result in increased synthesis and release of VP into the blood. This may increase peripheral resistance and at the same time sensitize the baro-receptor reflex to reduce HR more effectively and keep BP in the borderline range. It is of interest to emphasize here that the baro-receptor reflex is one of the key controllers of VP release[39]. However, in BHRs exhibiting chronically increased BP the baro-receptor reflex fails to suppress VP release into the circulation regardless increased sensitivity. This could be due to the resetting of the baro-receptor reflex to higher BP values and no changes in BEI (Table 1). The concomitant increase of BRS and of baro-receptor reflex operating range in borderline hypertension may lead to its exhaustion, when exposed to environmental stressors.

Our experiments suggest that vasopressin V1bR could be involved in the autocrine regulation of VP release in BHRs under baseline physiological conditions. V1bRs are abundantly expressed in the PVN and are co-localized with V1aR in VP secreting neurons, in both magnocellular and parvocellular divisions [6,40]. Gouzènes *et al* [6] provided evidence *in vivo* that VP released somato-dendritically by exocytosis, regularizes the firing pattern of MCNs to best fit the axonal release of VP to physiological demand. Vesicles containing VP prepared for somatodendritic release are positive for both V1aR and V1bR receptors. A number of reports

For Review Only

mention V1aR in autocrine regulation of VP release [41]. In rats, Hurbin *et al* [41] have shown that dehydration up-regulates the V1aR mRNA in MCNs and V1aR receptor content in exocytotic granules. They also noted a down regulation of V1aR mRNAs in MCNs, and V1aR receptor content in exocytotic granules during water over-load. Son and colleagues [8] further demonstrated that V1aRs located on neurons in the parvocellular division of PVN are stimulated by VP released by MCNs in the magnocellular division to provide an integrated humoral and neurogenic response to osmotic challenge. Ribeiro and colleagues [9] observed that V1aRs in the PVN are involved in the mediation of sympatho-excitation during salt load in normotensive rats. Lozić and coworkers [10] investigated the role of V1aRs in the PVN during stress and showed that they reduce BRS under baseline physiological conditions, and increase the neurocardiogenic stress response. Our present results do not indicate a change in V1aR expression in the PVN of BHRs under baseline conditions, when exposed to saline load and when exposed to saline load plus stress. Rather, our results point to increased expression of V1bR as a possible mechanism involved in autocrine positive feed-back control of VP release in BHR.

In the parvocellular division of the PVN, VP is expressed in a number of neurons that project to the brainstem vasomotor centre in the RVLM and to pre-ganglionic sympathetic neurons located in the IML column of the spinal cord, where V1aRs are found [5]. In clinical and experimental practice, sympathetic outflow to the blood vessels and the heart can be assessed indirectly by autonomic spectral markers [20]. In the present experiments no changes in LF-BP and LF/HF HR spectral markers were observed under baseline conditions in BHRs (Figure 2), suggesting that increased expression of VP and V1bR in the PVN occurs in the magnocellular division and does not alter baseline neurogenic cardiovascular control in BHRs.

The concept that hypertension derives from an interaction between genetic and environmental factors was first proposed by Folkow [42]. Of the environmental factors, dietary

For Review Only

sodium intake and environmental (psychosocial) stress are considered important. DiBona and Rios [43] have shown that intravenous isotonic saline infusion exaggerates natriuresis in SHR due to increased renal sympathetic nerve activity (RSNA) withdrawal. In SHR and Dahl rats Koepke and colleagues [44,45] have further shown that increased dietary sodium intake augments efferent RSNA in response to environmental stress leading to greater renal vasoconstriction and profound antidiuretic and antinatriuretic response. They have also shown that BHR rats exposed to high sodium intake have a response similar to SHR rats, whilst BHRs exposed to low sodium intake (up to 1% w/v) does not and respond like normotensive controls [46]. This is in line with our findings that BHRs drinking saline (corresponding to low sodium intake) exhibited sympathetic withdrawal, as documented indirectly by LF-BP spectral decrease, allowing effective natriuresis, as in normotensive rats. A decrease in V1bR in the PVN of salt loaded BHRs also suggests removal of positive feed-back autocrine control on VP synthesis/release. Altogether, these data exclude the possibility that hydromineral imbalance occurs in BHR exposed to saline load contributing to BP dysregulation.

In present experiments, BHRs drinking isotonic saline and exposed to heterotypic stressors for 24 weeks showed higher BP values with respect to baseline conditions and BHR controls throughout the observation period. The lack of statistical significance in DBP increase could be due to improved BP control by baro-receptor reflex since BRS increased over time, though partial habituation cannot be excluded. Compared to BHR controls, SBP, LF-SBP, LF-DBP and LF/HF-HR constantly increased to reach highest values by the end of the stress protocol. An increase of LF-BP and LF/HF HR in present experiments indicates increased sympathetic outflow respectively to the blood vessels [47,48] and the heart [48-51] suggesting that this is the mechanism underlying stress-induced systolic hypertension. The possibility that autonomic spectral markers reflect increased sensitivity of peripheral vasculature and cardiac

For Review Only

tissue to catecholamines is unlikely since saline load below 1% NaCl has been shown not to modulate cardiovascular tissue reactivity to catecholamines in the BHR [52].

Our results do not support a role of peripheral VP in stress-induced hypertension in BHRs. Stress did not affect the level of expression of V1bR and VP in the PVN in respect to baseline conditions. Moreover, in BHRs exposed to saline load and stress, there was a decrease in plasma VP. This is not an unexpected finding since stressors provoking strong emotional reactions have been reported to inhibit systemic VP secretion [53]. In our experiments, BHRs were exposed to air-jet stress that induces startle and a panic reaction. We also changed the housing of BHRs and introduced another emotional, novelty paradigm. The present experiments show that the combination of these two paradigms reduces VP secretion without affecting its mRNA steady-state abundance. Nonetheless we cannot exclude the possibility that stress-induced hypertension also supressed VP release. It is of note to mention that concomitant saline load with stress blunts the HPA axis responsiveness to stress [54].

The present study has a limitation that needs to be addressed. The main drawback is the lack of data on V1aR and V1bR receptor protein levels in the PVN of both WR and BHRs under different experimental conditions. V1aR and V1bR are G protein coupled receptors (GPCRs) and as such they are important regulators of cellular function. As GPCR mRNA level and protein do not necessarily correlate, detection at protein level is important. GPCRs can be identified either using specific radioligands or antibodies. However, suitable radioligands for V1aR and V1bR are missing, while the selectivity of antibodies is doubtful due to great structural homology (up to 85 %) between V1aR, V1bR and oxytocin receptors [55], all found in the PVN of rats. The lack of antibody selectivity to vasopressin and oxytocin receptors is not an exception, but rather a rule for GPCR antibodies [56]. And a number of reports discourage the use of antibodies for GPCR protein detection [57-62]. At present state of the art, it seems that the expression of mRNA is

the only reliable approach for GPCRs assessment. Thus, we have used this technology to quantify changes in the expression of the V1aR and V1bR receptors at the level of steady-state mRNA abundance (see Figure 1).

V1aR and V1bR cell surface density is dynamic and changes under different physiological conditions due to either changes in mRNA expression or receptor internalization involving β-arrestin or clathrin-coated vesicles, marking the end of peptide action [63]. Internalized receptors have different faiths. They can undergo degradation and reduces protein level or recycle to the cell surface without affecting the protein level. V1aR have been shown to recycle rapidly, and V1bR have been reported to be preponderantly located in the cytoplasm even unstimulated [64], and none are being degraded following internalization. Therefore, we believe that it is unlikely that the steady-state abundance of the V1aR and V1bR is not related to mRNA level in this study.

In conclusion, the present results show, for the first time, that over-expression of VP and V1bR genes in the PVN is a characteristic trait of the borderline hypertension in the rat. Overexpression of VP and of V1bRs in the PVN does not seem to modulate neurogenic control of the circulation in BHRs under baseline conditions, but is associated with increased plasma VP, that may act at the same time to increases peripheral resistance and to increase BRS preserving BP at borderline level. Exposure of BHRs to prolonged isotonic saline load and stress increased sympathetic outflow to blood vessels and the heart inducing overt hypertension. At the same time plasma VP decreased and the expression of VP, V1bR and V1aR genes in the PVN was unaffected. This suggests that sympathetic hyperactivity and not peripherally released VP underlies stress-induced hypertension in BHRs.

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AUTORS CONTRIBUTIONS

All authors contributed to design, performance, analysis, reporting and writing of the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest

For Review Only

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For Review Only

Figure 1. Plasma VP and VP, V1aR and V1bR in the PVN of WR and BHR under baseline conditions and following saline loading with or without stress. Note the increase of plasma VP, VP and V1bR gene expression in the PVN of BHRs under baseline conditions with respect to WRs. Saline decreased V1bR gene expression. Adding stress reduced plasma VP without affecting VP, V1aR and v1bR gene expression. In this and the following figures WR-Wistar rats; BHR-borderline hypertensive rats; VP-vasopressin; V1aR-vasopressin receptor type 1a; V1bR-vasopressin receptor type 1b. Values are mean of at least six rats ± s.e.m.. *p<0.05; **p<0.01vs. WR baseline; † p< 0.05; †† p<0.01 vs. BHR baseline; ‡‡ p<0.01 vs. BHR saline (One-Way ANOVA followed by *post hoc* Tukey).

Figure 2. Autonomic spectral markers in BHRs and WRs under baseline conditions. In this and the following figures LF SBP-low frequency systolic blood pressure spectral component; LF DBP-low frequency diastolic blood pressure spectral component; LF HR-low frequency heart rate spectral component; LF/HF HR–low frequency to high frequency heart rate ratio. Values are mean of at least six experiments ± s.e.m. (Student's T-test).

Figure 3. BP, HR and BRS in BHRs exposed to saline load without or with stress. Mark the increase in SBP and BRS in BHRs exposed to saline solution plus heterotypic stress. HR was decreased in BHRs drinking saline with or without exposure to stress. Values are mean of at least seven rats ± s.e.m.. *p<0.05, *** p<0.001 (ANOVA for repeated measures) vs. baseline; †p<0.05 vs. control BHR group (Hierarchical linear regression).

For Review Only

Figure 4. Autonomic spectral markers in BHRs exposed to saline load without or with stress. In saline loaded BHRs without exposure to stress LF SBP and LF DBP decreased over time. LF HR and LF/HF HR were lower compared to BHR controls. Note that LF SBP, LF DBP and LF/HF HR are higher in saline loaded plus stressed BHRs compared to BHR controls. Values are mean of at least seven rats ± s.e.m.. *$p<0.05$ (ANOVA for repeated measures) vs. baseline; †$p<0.05$; ††$p<0.01$ vs. control BHR group (Hierarchical linear regression).

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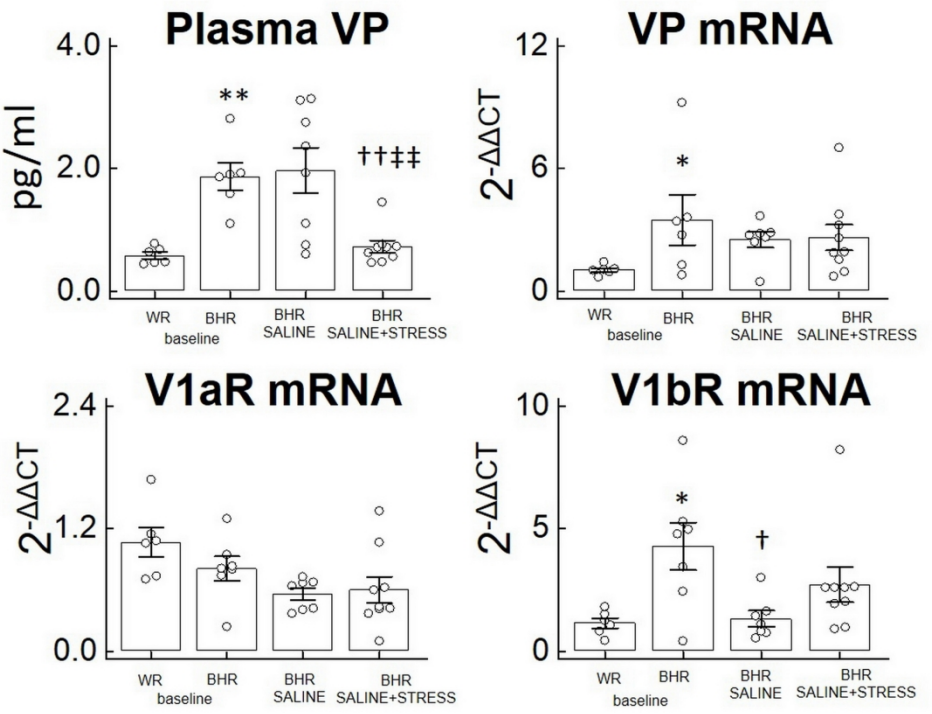
Table 1. BP, HR and baroreceptor reflex in BHRs and WRs under baseline conditions

	WR	BHR
SBP (mmHg)	115 ± 3	134 ± 3 **
DBP (mmHg)	82 ± 2	99 ± 2 **
HR (bpm)	321 ± 12	269 ± 6 ***
BRS (mmHg·ms)	1.1 ± 0.1	1.6 ± 0.2*
BEI	0.6 ± 0.03	0.63 ± 0.02
OR (ms·mmHg)	114.6 ± 36.5	205 ± 2.0 *
SP (mmHg)	105.1± 3.2	123.6±3.2**

Values are mean of experiments ± s.e.m.. SBP-systolic blood pressure; DBP-diastolic blood pressure; HR-heart rate; BRS-baro-receptor reflex sensitivity. BEI-baro-receptor effectiveness index; OR-operating range; SP-set point. **p<0.01, *** p<0.001 vs. WR.

For Review Only



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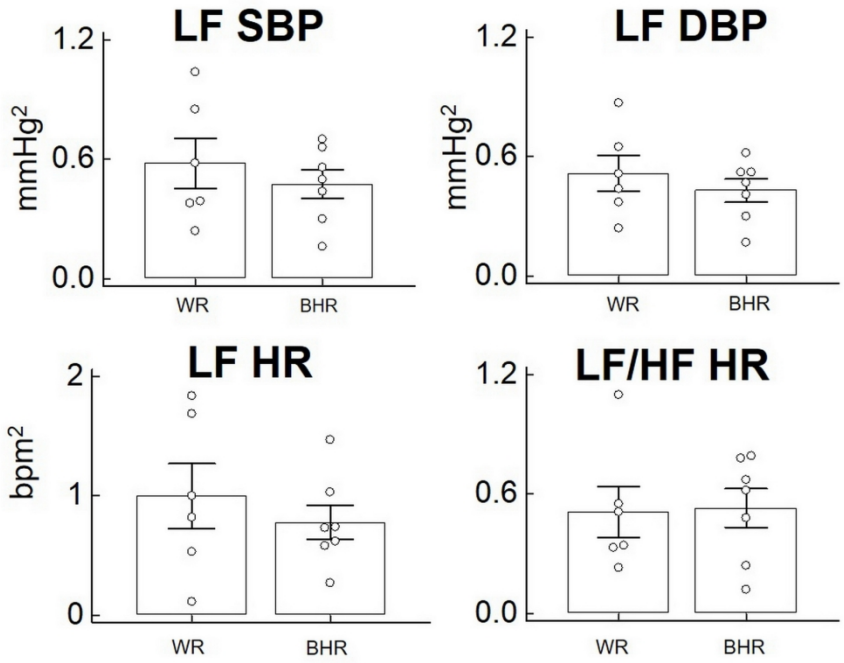

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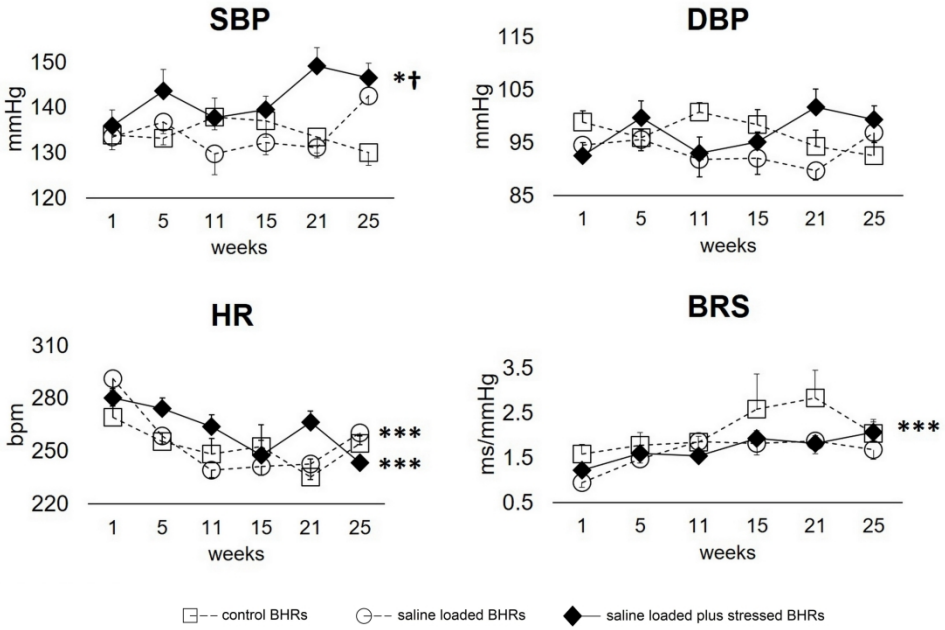

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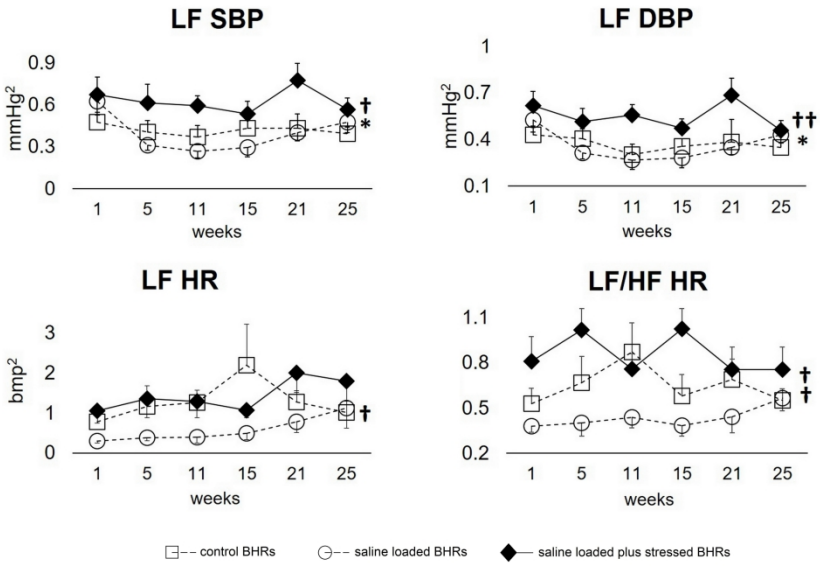

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308x195mm (300 x 300 DPI)